Detection and Determination of Adenosine Diphosphate and Related Substances in Plasma

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1. A method is described for detecting and determining the products of metabolism of ADP added to plasma at initial concentrations of about $1\mu\text{M}$ -ADP. 2. ATP, ADP, AMP, adenosine, inosine and hypoxanthine were detected in human platelet-rich plasma after incubation with ADP and in the presence of either heparin or heparin-citrate. 3. The products of incubation of ADP with human platelet-poor plasma in the presence of heparin were the same as with platelet-rich plasma, except that, when the initial concentration of ADP was $1.5\mu\text{M}$, little or no ATP was detected. 4. The ATP detected in platelet-rich plasma when $1.5\mu\text{M}$ -ADP was initially incubated was present in the platelets and not in the plasma. 5. The time for 50% decay of ADP in either platelet-rich or platelet-poor plasma in the presence of heparin was about 20min. when the initial concentration of ADP was $200\mu\text{M}$, but was 6–9min. when the initial ADP concentration was 1.5 – $2.5\mu\text{M}$. The corresponding values in the presence of heparin-citrate were about 45min. and about 9–12min. respectively. 6. Hypoxanthine accumulated to a greater extent in platelet-rich than in platelet-poor plasma after the addition of ADP. 7. After incubation for 15–20min. of either platelet-rich plasma or suspensions of washed platelets in saline with adenosine at an initial concentration of about 3 – $4\mu\text{M}$, ATP, ADP and AMP were detected in the platelets. Similar incubations of washed platelets with inosine also showed the formation of these substances, but to a much less extent. 8. After the addition of adenosine to suspensions of washed platelets in saline, inosine and hypoxanthine were detected in the incubation mixture. After the addition of inosine, hypoxanthine was detected. 9. When ADP at an initial concentration of $1.5\mu\text{M}$ was added to platelet-rich plasma containing adenosine deaminase, no adenosine was detected in the incubation mixture. There was no difference in the rate of decay of ADP in the presence or absence of the deaminase, but ATP formation was decreased in its presence.

ADP at concentrations of 0.2 – $1.0\mu\text{M}$ causes clumping of platelets when added to stirred platelet-rich plasma. On continued agitation, the clumped platelets disaggregate after a time varying with the amount of ADP added. After the addition of $0.2\mu\text{M}$ - or $1.0\mu\text{M}$ -ADP the time taken for dispersal of clumps of platelets in human plasma is about 2min. or 10min. respectively (Born & Cross, 1963; Cuthbertson & Mills, 1963b). Both AMP and adenosine can inhibit this effect of ADP in human platelet-rich plasma (Born & Cross, 1963). The experiments reported below were carried out to investigate the mode of breakdown of ADP in plasma, because the rate of destruction of ADP and the possible formation of AMP and adenosine

from it might play a part in the control of platelet aggregation.

A method was required that would determine ADP and substances formed from it in plasma at initial concentrations of 1 – $2\mu\text{M}$ -ADP. This was achieved by incubating radioactive ADP with plasma and subsequently passing a trichloroacetic acid extract of it through charcoal to adsorb the nucleotides, nucleosides and purines, which were then eluted. The adsorption of such compounds on charcoal has been used as a method of separating them from unwanted substances for preparative purposes (Hurlbert, 1957), and Threlfall (1957) adsorbed nucleotides on charcoal in an analytical procedure. The eluates were concentrated and the individual substances separated by paper chromatography and electrophoresis. A preliminary report

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of part of this work has been given (Ireland & Mills, 1964).

MATERIALS AND APPARATUS

Nucleotides, nucleosides and purines. ATP and ADP were obtained from Schwarz BioResearch Ltd., Mount Vernon, N.Y., U.S.A.; adenine, adenosine, inosine, hypoxanthine, ITP, IDP and IMP were from L. Light and Co. Ltd., Colnbrook, Bucks.; AMP was from British Drug Houses Ltd., Poole, Dorset. The ATP and ADP contained 2–5% of ADP and 2–5% of AMP respectively as material detectable under u.v. light after electrophoresis at acid pH. The inosine contained a trace of a substance detectable under u.v. light and having the mobility of adenosine on electrophoresis at acid pH (see the Methods section). The adenosine contained a trace of a substance detectable under u.v. light and having the mobility of adenine on electrophoresis at acid pH.

[8-¹⁴C]ADP, [8-¹⁴C]ATP, [8-¹⁴C]inosine and [8-¹⁴C]IMP, of specific activity 28, 28.2, 21.8 and 22mc/m-mole respectively, were obtained from Schwarz BioResearch Ltd. Electrophoresis under acid conditions showed that the [8-¹⁴C]ADP contained 2–3% of radioactivity as AMP and the [8-¹⁴C]ATP contained about 3% as ADP. [8-¹⁴C]-Adenosine of specific activity 14.7 and 28.4mc/m-mole was obtained from The Radiochemical Centre, Amersham, Bucks. It contained a trace of material that stayed at the origin and a trace of material that had the mobility of adenine on electrophoresis under acid conditions.

Enzymes, test kits and other reagents. Adenosine deaminase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany: 1mg. of adenosine deaminase was contained in 0.1 ml. of 2.8M-(NH₄)₂SO₄. The enzyme kit ADP/AMP-15980, used for determining ADP and AMP with pyruvate kinase, lactate dehydrogenase and myokinase, was also obtained from C. F. Boehringer und Soehne G.m.b.H. Heparin was obtained from Evans Medical Ltd., Speke, Lancashire, and contained 145.2 i.u. of heparin/mg. Vitamin B₁₂ was obtained from Glaxo Laboratories Ltd., Greenford, Middlesex. EDTA was supplied by Hopkin and Williams Ltd., Chadwell Heath, Essex, as the disodium salt. Other reagents were of A.R. grade except for butan-1-ol used for chromatography, which was technical grade obtained from the Distillers Chemical Co. Ltd., London. Trichloroacetic acid (Hopkin and Williams Ltd.) was dissolved in water and filtered through Whatman no. 42 filter paper to remove a slight turbidity. Deionized water was used throughout.

Salt solutions. Saline solutions unless otherwise stated contained CaCl₂, MgSO₄, NaCl and KCl at concentrations of 2.6, 1.2, 145 and 48mm respectively. Citrate solutions contained 3.13g. of trisodium citrate/100 ml. of water. The pH of this solution was about 7.4. Buffered 0.9% NaCl solution, pH 7.4, with and without EDTA was prepared as described by Haslam (1964).

Charcoal-kieselguhr columns. Glass columns (38 cm. long × 7 mm. internal diam.), with a capacity of about 11 ml. and with one end suitably narrowed, were plugged with a small wad of absorbent cotton wool. A kieselguhr bed (Celite 535; Johns-Manville Ltd., London), about 5–6 mm. in height, was packed down by water washing on top of the cotton wool. A slurry of 20 mg. of charcoal (Darco G.60; Atlas Powder Co. Ltd., Wilmington, Del., U.S.A., supplied

by Honeywell and Stein Ltd., London) and 40 mg. of kieselguhr in water was next added and packed down with water washing to give a bed about 6 mm. in height. Each column was then washed successively with 10 ml. of 10% (w/v) trichloroacetic acid, 10 ml. of water, 10 ml. of eluate solution containing ethanol–water–0.57% (v/v) aq. ammonia (sp.gr. 0.88) (30:19:1, by vol.) and 10 ml. of water.

Ultraviolet lamp. The u.v. light of wavelength mainly 254 mμ was provided by a Chromatolite lamp (Hanovia Ltd., Slough, Bucks.). This was used to detect areas of nucleotides, nucleosides and purines after chromatography or electrophoresis on paper.

Evaporator. This was a rotary evaporator (Wright Scientific Ltd., London) attached to a mains-water vacuum pump. The flask containing the solution to be evaporated was rotated in a water bath kept at 37°.

Evaporation flasks. Round-bottomed flasks, 50 ml., with the 'shoulders' flattened out, were cleaned overnight in chromic acid, washed with water, dried and then treated with silicone fluid [2% (v/v) solution of dichlorodimethylsilane in carbon tetrachloride (Repelcote; Hopkin and Williams Ltd.)], which was removed after a few minutes. The flasks were then dried and washed with water. After being redried the flasks were ready for use.

Chromatography and electrophoresis. Chromatography was performed downward in conventional equipment and electrophoresis was carried out in the apparatus of Mills & Pegler (1961). Whatman no. 541 double-acid-washed paper was used (W. and R. Balston Ltd., London).

Spectrophotometers. A Unicam SP.500 and a double-beam automatic recording Beckman spectrophotometer were used.

Glassware. All flasks, centrifuge tubes and pipettes used for blood or plasma were treated with silicone as described. Glass syringes were lightly coated internally with silicone fluid MS200/100 centistokes (Hopkin and Williams Ltd.).

Micropipettes. Drummond microcap pipettes (supplied by Shandon Scientific Apparatus Ltd., London) containing 10 μl. were used for application of aqueous solutions to paper. Calibrated microsyringes [Scientific Kit Co., Washington, D.C., U.S.A., supplied by Scientific Industries International Inc. (U.K.) Ltd., London] to hold 10 μl. were used for adding aqueous solutions to incubation mixtures. Volumes greater than 10 μl. were added to incubation mixtures by means of constricted auto-zero pipettes delivering either 20 μl. or 50 μl. of aqueous solution (H. J. Elliot Ltd., Treforest, Glamorgan).

Detection and estimation of radioactivity. Radioactivity was detected on paper by means of X-ray Curix film supplied by Gevaert Ltd., Slough, Bucks. Radioactivity was estimated in a scintillation counter (N664A) supplied by Ekco Ltd., Southend-on-Sea, Essex, and supported by automatic counting equipment (AC/300/6) supplied by Panax Equipment Ltd., Redhill, Surrey.

Nephelometer. The aggregation and disaggregation of platelets was measured in an automatic recording nephelometer as described by Cuthberton & Mills (1963a).

METHODS

Preparation of human plasma. By syringe 19 ml. of blood was taken from the antecubital vein of male volunteers and added to 1 ml. of saline solution containing heparin, to give a final concentration of 10 i.u. of heparin/ml. of

blood. The heparin-treated blood was centrifuged to give either PR-plasma* (165g for 10 min.) containing 170×10^6 – 350×10^6 platelets/ml. of plasma or PP-plasma (2500g for 10 min.) containing 3×10^6 – 40×10^6 platelets/ml. The plasma was sometimes further diluted with either 2 ml. of saline solution or 2 ml. of citrate solution added to 9 ml. of plasma. This dilution is noted as 'diluted 3:19' in Figs. 2, 3 and 4.

Isolation of human platelets. To 1.5 ml. of 77 mM-EDTA, pH 7.4, 18.5 ml. of blood was added. After centrifuging the blood at room temperature for 10 min. at 165g, the PR-plasma was again centrifuged at 165g for 3 min. and the platelets in the supernatant were then isolated, washed and resuspended at 2–4° as described by Haslam (1964).

Preparation of rat plasma. Male rats, anaesthetized with ether, were bled from the inferior vena cava into glass syringes coated with silicone fluid and containing heparin solution. To 9.5 ml. of blood 100 i.u. of heparin in 0.5 ml. of 0.9% NaCl solution was added. The heparin-treated blood was centrifuged at 372g for 10 min. to give PR-plasma containing 400×10^6 – 600×10^6 platelets/ml. of plasma.

Platelet counting. Plasma was diluted 1:10 with 3.13% (w/v) trisodium citrate and counted in Neubauer counting chambers by means of phase-contrast microscopy.

Incubation and treatment of samples. Two methods were used.

(a) The appropriate plasma or washed-cell suspension in a silicone-treated glass tube was mechanically shaken in a water bath at 37°. The substance to be incubated, i.e. ADP, [8- 14 C]ADP, [8- 14 C]adenosine or [8- 14 C]inosine, was added to the plasma or washed-cell suspension, in either saline or aqueous solution and in a volume not greater than 1% of the volume of the incubation mixture. The initial concentration of the radioactive compounds added ranged from 0.6 to $3.3 \mu\text{M}$, but when non-radioactive ADP was added the initial concentration ranged from 100 to $200 \mu\text{M}$.

After incubation of the radioactive compounds at 37° for the desired time, a known portion of the incubation mixture, usually 0.5 ml. or 1 ml., was removed and added to 4.5 ml. of ice-cold 10% (w/v) trichloroacetic acid, which contained approx. $30 \mu\text{M}$ moles of each of carrier ATP, ADP, AMP, adenosine, inosine and hypoxanthine/4.5 ml. of trichloroacetic acid (see the Experimental and Results section). This carrier solution was freshly made up for each experiment from stock solutions of the compounds listed and was kept at 0°. The trichloroacetic acid–plasma or trichloroacetic acid–cell-suspension mixture was centrifuged at 0° (12000g for 10 min.) to give a protein precipitate and a clear supernatant.

After incubation of non-radioactive ADP with PR-plasma, portions of the incubation mixture were placed in cold polythene centrifuge tubes and immediately centrifuged at 0° and 3500g for 2 min. Of the resulting supernatant 1 ml. was then added to 4.5 ml. of 10% (w/v) trichloroacetic acid and treated as described above. This centrifugation was necessary to remove platelets, from which ATP and ADP are released on treatment with trichloroacetic acid.

(b) To the non-silicone-treated glass tube of the nephelo-

meter 0.5 ml. of PR-plasma was added and stirred with a glass rod connected to an electric motor and rotated at 1000 rev./min. The temperature of the water in the jacket surrounding the glass tube was kept at 37°. After incubation with added [8- 14 C]ADP for the desired time, about 1 ml. of 10% (w/v) trichloroacetic acid containing the carrier compounds was added to the incubation tube to stop the reaction, and the tube was then quantitatively rinsed with the remainder of the 4.5 ml. of trichloroacetic acid–carrier solution to give the final mixture, which was centrifuged to give a protein precipitate and supernatant.

Charcoal adsorption and elution. The procedure was carried out in the cold room (2–4°). A known volume of each trichloroacetic acid–plasma supernatant was applied to each column. Generally 4.7–5.0 ml. was applied, and this volume passed through the column in about 30–40 min. This was washed immediately with 5 ml. of cold water, and the effluent solution and water wash were rejected. Then 10 ml. of cold eluate solution was used. The eluate was collected in silicone-treated flasks, evaporated to dryness in a vacuum rotary evaporator and kept in the flasks at –20° until required. Before chromatography or electrophoresis the residue was dissolved in either 50 μl . of water or two successive 20 μl . portions of water, by rotating the flask so that the whole glass surface was washed by the droplet of water.

Separation and estimation of metabolites. Of each 50 μl . of final eluate a 20 μl . portion was spotted on to paper in two 10 μl . portions. Each pipette was rinsed with 10 μl . of water after each application of test solution, and these washes were also applied to the paper. If the whole contents of the evaporation flask were required on paper, this was achieved by successively washing the flask with two 20 μl . portions of water and applying the whole in four 10 μl . portions. Samples were applied 4 cm. apart, and each application was allowed to dry before the next was applied.

Electrophoresis was done in the cold (2–4°) with cold 1.5M-formic acid–2.0M-acetic acid mixture (1:1, v/v), pH 1.9 (Efron, 1960); a voltage of 500 v was used for 2 hr. at a voltage gradient of about 25 v/cm. Vitamin B₁₂ was applied to the papers to establish the electroosmotic flow. In preliminary experiments two other systems were used, 0.02M-citric acid–sodium citrate, pH 3.5 (Thomson, 1960), and 0.05M-sodium borate, pH 9.2 (Efron, 1960). After electrophoresis the paper was air-dried in the cold room by careful application of an air stream from a hair-drier. Hot air was avoided. This precaution was, however, unnecessary when only the radioactive substances were being studied, and the papers containing them were dried with application of warm air. Chromatography was done at room temperature with the following solvent systems: solvent 1, butan-1-ol–acetone–acetic acid–5% (v/v) aq. ammonia (sp.gr. 0.88)–water (9:3:2:2:4, by vol.) (Randerath & Struck, 1961); solvent 2, isobutyric acid–N-ammonia–0.1M-EDTA (100:60:1:6, by vol.) (Cain, Kushmevick & Davies, 1963). Development was downward for 5–6 hr. The chromatograms were dried at room temperature.

Papers not containing radioactive substances were viewed under u.v. light; the relevant areas were outlined in pencil and cut out as well as areas of paper not containing applied material that served as blanks. They were then cut into small pieces and eluted by shaking for 1 hr. with 3 ml. of water. To those tubes containing eluates of ATP, ADP, AMP and adenosine 50 μl . of 0.25N-NaOH was added

* Abbreviations: PR-plasma, platelet-rich plasma; PP-plasma, platelet-poor plasma.

to ensure a pH between pH7 and 9. The pH of eluates containing inosine and hypoxanthine were not adjusted, the pH being between pH5 and 6. The eluates were centrifuged to remove paper fibres, and the extinctions of the clear eluates were recorded at the appropriate wavelength for each solution, with a water blank. Data on wavelengths and extinction coefficients for each compound at the appropriate pH were obtained from Burton (1959), and the concentration of each compound was calculated/ml. of incubation mixture after correction for blank values. In one experiment ATP, ADP, AMP and adenosine were eluted from the paper with 0.01N-HCl. These solutions were read as described above, against a 0.01N-HCl blank, and the appropriate factors were applied (Burton, 1959) to obtain the concentration of each compound.

Papers containing radioactive substances were viewed under u.v. light, and the positions of the carrier substances were outlined in pencil. The paper was then placed on X-ray film, and after the appropriate exposure, usually between 16 and 60 hr., the film was developed and fixed. The film was superimposed on the paper, and the positions of the radioactive areas were compared with those of the carrier substances. Each radioactive area was then cut out in a rectangle 2 cm. $\times$ 2.5 cm. and placed flat in the counting cell; 1 ml. of a solution containing 0.75 g. of 2,5-diphenyl-oxazole and 0.075 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/250 ml. of toluene was added. The counting efficiency was about 50% at the extra-high-tension and discriminator voltages used, 1100 v and 34.5 mv respectively.

Calculation of results. Radioactivity was recorded as counts/min. Known amounts of the radioactive compound used for the relevant incubations were also submitted to chromatography or electrophoresis along with test samples and were counted, as were areas of paper not containing radioactivity. The results of the latter served as blank values and were subtracted from the relevant test and standard values. The values so obtained were expressed as

counts/min./substance/ml. of incubation mixture and were not corrected for recovery of the relevant substances from charcoal unless stated otherwise.

The results obtained for any given substance at a particular incubation time were generally expressed as percentages of the total radioactivity recovered for that particular incubation time.

Determination of ADP and AMP by enzyme assay. ADP and AMP were determined by the use of pyruvate kinase, lactate dehydrogenase and myokinase, as described in the appropriate test kit, on HClO₄-soluble extracts of portions of the incubation mixtures.

Preparation of [8-¹⁴C]adenine and [8-¹⁴C]hypoxanthine. These compounds were prepared by hydrolysis of [8-¹⁴C]-adenosine of specific activity 28.4 mc/m-mole and [8-¹⁴C]-inosine respectively in 0.5N-HCl for 1 hr. at 100°. The resulting solutions, which were shown by electrophoresis to be free of radioactivity other than that due to the adenine or hypoxanthine formed, were used for the charcoal recovery experiments.

EXPERIMENTAL AND RESULTS

Recovery of ADP and related compounds from charcoal. The recoveries of approximately 200, 100 and 30 μ mole amounts of various reference compounds, with each compound applied separately to charcoal, are shown in Table 1. The recovery of all the compounds, except ITP, IDP and IMP, tended to decrease with decreasing amounts applied and this was particularly marked for adenine and hypoxanthine. The recovery of IMP remained constant with decreasing amounts applied, but that of ITP and IDP increased with decreasing amounts applied. More than 55% of both these compounds

Table 1. *Recovery of known amounts of ADP and related compounds on charcoal*

Approx. 24 mm solutions of each compound in either water (nucleotides and nucleosides) or 0.1N-HCl (purines) were prepared, and the concentration of each was determined by absorption spectrophotometry. Of each solution 10–40 μ l. was added to 10 or 20 ml. of a solution containing 4.5 parts of 10% (w/v) trichloroacetic acid and 1 part of 0.9% NaCl. Of these solutions 1–4 ml. was applied to the appropriate charcoal column. After a water wash, elution and evaporation of the eluate to dryness, the residue was dissolved in 4 ml. of water and the concentration of each compound determined by absorption spectrophotometry. Results were corrected for blank values obtained from eluates of charcoal to which no compounds had been applied. Results given are the means of two or more determinations except those for ATP and inosine, which were from single determinations.

Compound Expt.	Amount applied (μ moles)			Amount recovered (% of amount applied)		
	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>b</i>	<i>c</i>
ATP		103.0	38.6		85	81
ADP	197.4	100.6	35.7	89	90	75
AMP	207.6	102.6	38.5	96	91	86
Adenosine	205.7	96.4	30.5	89	75	69
Adenine	193.3	102.1	33.7	58	39	23
ITP	199.7	99.8	26.7	11	14	42
IDP	198.7	99.3	28.4	15	31	64
IMP	206.4	103.4	36.5	68	76	72
Inosine	203.0	119.3	37.3	98	82	74
Hypoxanthine	190.0	104.2	36.8	83	70	39

after application of 200 μ mole amounts to charcoal was found in the combined effluent and water-wash fraction after ether extraction to remove the trichloroacetic acid.

With small constant amounts of radioactive ATP and ADP in the presence of various amounts of non-radioactive ATP and ADP respectively, it was shown (Table 2) that the recovery of these compounds decreased by some 6–12% when the

amount of each compound applied to charcoal was decreased from 200 μ moles to 97 or 33 μ moles. Table 2 also shows that about 0.7% of the applied ADP was converted into AMP and that about 2.8% of the applied ATP was converted into ADP after charcoal treatment and electrophoresis.

The results given in Table 3 show the recoveries obtained when 21–33 μ mole carrier amounts of each of ATP, ADP, AMP, adenosine, adenine,

Table 2. *Recovery of radioactive ATP and ADP from charcoal in the presence of various amounts of carrier ATP and ADP respectively*

Expt. A. Portions (5 ml.) of 8% (w/v) trichloroacetic acid, each containing 0.4 μ mole of [8-¹⁴C]ADP, and the amounts of non-radioactive ADP indicated were applied to charcoal. After a 10 ml. water wash, the eluates were treated as described in the text and submitted to electrophoresis. Results are derived from the values for radioactivity obtained after electrophoresis of the charcoal eluates and known amounts of untreated [8-¹⁴C]ADP. The [8-¹⁴C]ADP solution contained 2.8% of the radioactivity as AMP before charcoal treatment. Expt. B. Portions (5 ml.) of plasma-trichloroacetic acid extract (supernatant from 5 ml. of plasma and 22.5 ml. of 10% trichloroacetic acid mixture), each containing 1.03 μ moles of [8-¹⁴C]ATP, and the amounts of non-radioactive ATP indicated were applied to charcoal. Subsequent treatment was as above except that the water wash was 5 ml. and results are calculated from known amounts of untreated [8-¹⁴C]ATP. The [8-¹⁴C]ATP solution contained 4.7% of the radioactivity as ADP before charcoal treatment.

Radioactive compound	Carrier content (μ moles)	Radioactivity recovered (%)	AMP content of recovered radioactivity (%)	ADP content of recovered radioactivity (%)
Expt. A: ADP	196	93	3.0	
	97	81	3.0	
	32	87	3.1	
	11	82	3.5	
Expt. B: ATP	200	88		6.2
	100	80		6.9
	33	82		7.5
	11	74		7.3

Table 3. *Recovery of radioactive compounds on charcoal in the presence of standard amounts of carrier compounds*

To 5 ml. amounts of 10% (w/v) trichloroacetic acid–0.9% NaCl solution (4.5:1, v/v), containing carrier compounds, were added 10–40 μ l. amounts of each of the radioactive compounds. Portions (4.9 ml.) of the resulting solutions were applied to charcoal and, after a water wash, elution and evaporation, the residue was applied to paper and submitted to electrophoresis. The appropriate carrier area, detected under u.v. light, was cut out and counted. Initial radioactivity applied to charcoal was calculated from counts obtained after electrophoresis of standard solutions of the radioactive compounds in the presence of their relevant carrier compounds. Results are corrected for background count of paper not containing applied radioactivity and the values given are means $\pm$ s.d. Numbers in parentheses refer to the numbers of determinations made. The content of the carrier solution applied to charcoal (μ moles/4.9 ml.) was: ATP, 24; ADP, 29; AMP, 24; adenosine, 26; adenine, 24; IMP, 21; inosine, 26; hypoxanthine, 23; total, 207.

Radioactive compound	Amount of radioactive compound applied/charcoal column		Amount recovered	
	(μ moles)	(counts/min.)	(counts/min.)	(%)
ATP	0.14	4402 $\pm$ 468 (8)	3410 $\pm$ 433 (10)	77
ADP	0.12	5297 $\pm$ 521 (8)	4475 $\pm$ 350 (10)	84
Adenosine	0.17	6035 $\pm$ 405 (8)	4272 $\pm$ 373 (10)	71
Adenine	0.15	5293 $\pm$ 136 (4)	480 (2)	9
IMP	0.16	5335 $\pm$ 180 (4)	2926 $\pm$ 170 (4)	55
Inosine	0.18	4673 $\pm$ 404 (8)	4016 $\pm$ 367 (10)	86
Hypoxanthine	1.40	36162 $\pm$ 2682 (3)	26542 $\pm$ 1352 (4)	73

IMP, inosine and hypoxanthine and small amounts of radioactive ATP, ADP, adenosine, IMP, inosine, hypoxanthine and adenine were applied to charcoal together in one solution. In similar experiments adenine and IMP were omitted and the amount of each of the following carrier compounds applied to charcoal was (μmoles): ATP, 29; ADP, 36; AMP, 30; adenosine, 32; inosine, 32; hypoxanthine, 31; total, 190; the recovery of radioactive ATP, ADP, adenosine, inosine and hypoxanthine was not significantly different from the values given in Table 3. Although AMP carrier was included in these experiments radioactive AMP was not included and the recovery of AMP in such systems is not known. However, the results shown in Fig. 1

Fig. 1. Comparison of results of determining (a) AMP and (b) ADP by two methods. The ADP and AMP contents of various incubation mixtures (see the text) were determined by the charcoal-chromatography and electrophoresis method (charcoal method), and also by an enzyme-linked oxidation of NADH method (enzyme method). The slopes of the curves given, determined by the method of least squares, are 0.844 for AMP and 0.904 for ADP.

indicate that AMP is recovered in about 84% yield after charcoal treatment when about 40–100 μmoles of AMP is present in solutions also containing ADP, ATP, adenosine, inosine and hypoxanthine in a total amount of about 200 μmoles .

Chromatography and electrophoresis of ADP and related compounds. The mobilities and R_F values of various reference compounds in one electrophoretic and two chromatographic systems are given in Table 4.

Elution from paper with water or 0.01N-hydrochloric acid, of each of ADP, AMP, adenosine, inosine and hypoxanthine in amounts varying from 35 to 52 μmoles of compound after electrophoresis gave recoveries greater than 94%.

Little degradation or loss of ADP on the paper took place under the acid conditions of electrophoresis used. This was demonstrated by applying 1.5 μmoles of $[8-^{14}\text{C}]\text{ADP}$ (total count, 47.64×10^3 counts/min.; $[8-^{14}\text{C}]\text{AMP}$ content, 2.3%) to two papers, submitting one paper to one-dimensional electrophoresis for 2 hr. and submitting the other to two-dimensional electrophoresis for 2 hr. in each direction. The latter paper was not dried after the first electrophoresis, but was turned through 90° and submitted to electrophoresis again. The radioautographs showed no streaking; the amount of $[8-^{14}\text{C}]\text{ADP}$ found after the two-dimensional electrophoresis was 2% less than that found after the one-dimensional electrophoresis.

Amounts of 1 μmole of $[8-^{14}\text{C}]\text{ATP}$ did not

Table 4. Paper chromatography and paper electrophoresis of reference compounds on Whatman no. 541 paper

The R_F values given are intended as a guide to the relative positions of the reference compounds. Variation of R_F values occurred with different batches of paper. The temperature of chromatography was 20–26°, and that of electrophoresis was 2–4°. Compositions of solvent systems and conditions are given in the Methods section. The mobilities of compounds are given relative to that of vitamin B₁₂; + indicates towards the anode, – indicates towards the cathode.

	R_F		Mobility in formic acid-acetic acid, pH 1.9 (cm.)
	In solvent system 1	In solvent system 2	
ATP	0.13	0.37	+3.7
ADP	0.19	0.49	+1.9
AMP	0.32	0.60	–0.2
Adenosine	0.54	0.85	–3.9
Adenine	0.58	0.91	–6.8
ITP	0.10	0.17	+4.8
IDP	0.14	0.23	+3.6
IMP	0.23	0.33	+1.9
Inosine	0.46	0.57	–0.2
Hypoxanthine	0.53	0.66	–3.0

migrate during electrophoresis. Most of the radioactivity was found to be on or near the point of application. However, in the presence of 30–200 μ M of non-radioactive ATP, 1 μ M of [8- 14 C]-ATP migrated without more than a trace of radioactivity being left on the origin. About 1 μ M of [8- 14 C]ATP (total count, 35.88×10^3 counts/min.; [8- 14 C]ADP content, 7.6%) in the presence of 160 μ M of non-radioactive ATP was applied to two papers, which were then submitted to one- and two-dimensional electrophoresis, as described for [8- 14 C]ADP above. The radioautographs showed no streaking; the amount of [8- 14 C]ATP found after the two-dimensional electrophoresis was 1% less than that found after the one-dimensional electrophoresis.

The two separation methods most used were electrophoresis in formic acid-acetic acid, pH 1.9, and chromatography with solvent system 1 (Table 4). The systems were complementary, since the electrophoresis did not separate AMP from inosine, whereas the chromatography did not separate adenosine from hypoxanthine. Table 5 gives results of comparing these two methods. Lower values for the amounts of ATP and for adenosine were obtained after electrophoresis than after chromatography, but the reverse occurred for ADP and for the total amounts of AMP and inosine found. Chromatography for 6 hr. does not always completely separate ATP from ADP, and some ADP may have been determined as ATP. It is also possible that small amounts of hypoxanthine were determined along with the adenosine after chroma-

tography. Hypoxanthine not detected by radioautography after the electrophoresis was not determined in this experiment.

Identification of products of incubation of ADP with human plasma. On the basis of chromatography in two solvent systems and electrophoresis in one system (see Table 4 and the Methods section), the products of incubation of about 200 μ M amounts of ADP added to human PR-plasma or PP-plasma with either heparin or citrate as anticoagulant were identified as ATP, ADP, AMP, adenosine, inosine and hypoxanthine. No IMP, IDP, ITP or adenine was detected.

After incubation of ADP, at initial concentrations of about 200 μ M, with PR-plasma for various times, the separated substances were eluted with water from paper, and the eluates were submitted to u.v. spectroscopy. The substances thought to be ATP, ADP, AMP and adenosine showed absorption maxima at 257–260 $m\mu$ in acid or neutral solution. The substance thought to be inosine showed a maximum at 248.5 $m\mu$ in acid solution (pH 4.4) and a maximum at 253 $m\mu$ in alkaline solution (pH 11.2). The substance thought to be hypoxanthine showed a maximum at 250 $m\mu$ in acid solution (pH 3.9) and a maximum at 258–259 $m\mu$ in alkaline solution (pH 11.0). The results agree with the maxima given by Burton (1959) for these substances.

The ADP and AMP contents of mechanically shaken incubation mixtures at various times after the addition of ADP were determined by the method given and also by the specific oxidation of NADH in perchloric acid-soluble extracts of plasma by the use of the enzymes pyruvate kinase, lactate dehydrogenase and myokinase. The incubation mixtures contained either PR-plasma or PP-plasma, with either heparin or heparin-citrate as anticoagulant, and ADP was added at an initial concentration of about 200 μ M. The results of comparing the two methods are given in Fig. 1. The curves illustrated in Fig. 1 were calculated by the method of least squares and were assumed to pass through the origin.

The substance detected in platelets after incubation with [8- 14 C]adenosine, thought to be [8- 14 C]-ATP on the basis of its mobility on electrophoresis and R_f value on chromatography (see below), was isolated and further identified. To 5 ml. of human PR-plasma containing heparin was added 50 μ l. of an aqueous solution containing 36.5 μ M of [8- 14 C]adenosine of specific activity 14.7 mc/mole. The mixture was incubated for 45 min. at 37° with mechanical shaking, and the reaction was stopped by adding 22.5 ml. of 10% (w/v) trichloroacetic acid containing about 150 μ M of each of ATP, ADP, AMP, adenosine, inosine and hypoxanthine as carrier substances. After centrifugation, five 5 ml. amounts of the supernatant were

Table 5. Comparison of results of determination of radioactive substances after separation by both chromatography and electrophoresis

Of three different eluates each containing the radioactive products of incubation of [8- 14 C]ADP with PR-plasma, 20 μ l. amounts were submitted to (a) electrophoresis and (b) chromatography. The mean results of the three determinations are expressed as counts/min. found for each substance. Correction has been made for background count of similar areas of paper not containing applied radioactivity. Solvent system 1 (Table 4) was used for the chromatography, and the electrophoresis solution was as indicated in Table 4.

	(a)	(b)	$\frac{(b)}{(a)} \times 100$
ATP	486*	579*	119.1
ADP	2438	2118	86.9
AMP	966	{ 456	870
Inosine		{ 414	
Adenosine	1150	1229	106.9
Hypoxanthine	—		
Total	5040	4796	95.2

* Mean of two determinations.

each put on charcoal columns, which were washed with water and eluted. The concentrated eluates were submitted to electrophoresis; after careful drying of the paper, the carrier ATP areas were outlined with pencil under u.v. light, then cut out and all the paper containing ATP was added to 3ml. of water containing about 10 μ l. of 2N-sodium hydroxide to bring the pH of the mixture to about pH 5. These operations were done in the cold. After 30min. shaking at room temperature, paper and supernatant were separated by centrifugation, and the paper was washed with 2ml. of water and re-centrifuged. To the combined supernatant containing the radioactive substance and about 120m μ -moles of carrier ATP were added a further 700m μ -moles of ATP. The mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in 2ml. of water; 2ml. of 0.154N-hydrochloric acid was added, the solution being kept at room temperature (22–26°). Samples (1ml.) were withdrawn 41, 113 and 280hr. after adding the acid. To each 1ml. sample was added 4.5ml. of 10% trichloroacetic acid, and the whole volume was put on charcoal. After a water wash and elution the concentrated eluate was submitted to electrophoresis, and after radioautography the areas containing radioactivity were cut out and counted.

A similar acid hydrolysis was carried out with authentic [8-¹⁴C]ATP in the presence of ATP carrier. The radioactive substance isolated from the incubation of [8-¹⁴C]adenosine with PR-plasma and authentic [8-¹⁴C]ATP behaved similarly. There was a slow decay of [8-¹⁴C]ATP, with a half-life of about 70hr. Substances with the mobility on electrophoresis of [8-¹⁴C]ADP and [8-¹⁴C]AMP appeared and increased with time. The radioactivities recovered, not corrected for loss on charcoal treatment and expressed as a percentage of the total radioactivity recovered, attributable to ATP, ADP and AMP after 41hr. hydrolysis were 66, 32 and 1.5% respectively; after 113hr. they were 26, 68 and 5.4% respectively, and after 280hr. they were 3, 81.4 and 15.4% respectively. The results obtained for the test substance were within 4% of those for authentic [8-¹⁴C]ATP, and the values given are the means of the results of the authentic ATP and test determination. No other radioactive or non-radioactive substances other than those attributable to ATP, ADP and AMP were detected.

Incubation of ADP with human plasma containing heparin or heparin-citrate. During the incubation of ADP at an initial concentration of 200 μ M with PR-plasma for up to 4hr. it was found that ADP decayed approximately exponentially with a half-life of 20.8 ± 1.6 min. (s.d.) when heparin was the anticoagulant, but with heparin-citrate as anti-

coagulant the half-life for elimination of the ADP was 45.1 ± 1.5 min. (s.d.). AMP accumulated to reach a maximum of 46% of the added ADP by 30min. in the presence of heparin, after which it decayed, with little remaining at 120min. In the presence of heparin-citrate AMP accumulated to about 55% of the added ADP at 60min. and did not disappear until after 240min. These results were obtained when using the specific oxidation of NADH to estimate ADP and AMP (Ireland & Mills, 1964). By the charcoal and chromatography-electrophoresis method ATP, adenosine, inosine and hypoxanthine as well as ADP and AMP were detected and determined. The results are illustrated by Figs. 2 and 3.

Fig. 4 illustrates the results of an incubation of [8-¹⁴C]ADP at an initial concentration of 1.2 μ M-ADP with PR-plasma containing either heparin or heparin-citrate for up to about 20min. In three such comparisons, one with PR-plasma (Fig. 4) and two with PP-plasma, each plasma being obtained from a different man, the mean half-life for elimination of the added ADP was 8.0min.

Fig. 2. Incubation of ADP added to PR-plasma containing heparin to give an initial concentration of 140–200 μ M-ADP. Each point is the mean of three results obtained from three different plasmas at different times. Two of the plasmas were diluted with heparin (1:19, see the text), and one with heparin-saline (3:19, see the text). Incubation was at 37° with mechanical shaking. After the times indicated, each incubation mixture was worked up as described in the text. Portions of each final eluate from charcoal were submitted to both chromatography with solvent system 1 and electrophoresis (Table 4) and the extinctions of the various substances eluted from paper were determined. The horizontal straight line indicates the initial amount of ADP. The small horizontal lines indicate the ranges of each three results obtained. ●, ADP; ○, AMP; △, adenosine; ▲, inosine; □, hypoxanthine; ■, total. A small amount of ATP was detected at 30min. incubation time, but not determined. Results are not corrected for recovery of substances from charcoal.

Fig. 3. Incubation of ADP added to PR-plasma containing heparin-citrate to give an initial concentration of 140–190 μM . Conditions were as given in Fig. 2, except that all plasmas were diluted 3:19 (see the text) with heparin-citrate. ●, ADP; ○, AMP; △, adenosine; ▲, inosine; □, hypoxanthine; ■, total. A trace amount of ATP was detected at 30 min. incubation time, but not determined. Results are not corrected for recovery of substances from charcoal.

Fig. 4. Incubation of $[8-^{14}\text{C}]$ ADP added to PR-plasma containing either heparin-saline or heparin-citrate (dilution 3:19, see the text). To each of eight 1 ml. amounts of plasma containing the appropriate anticoagulant were added 10 μl . amounts of aqueous $[8-^{14}\text{C}]$ ADP solution to give an initial concentration of 1.2 μM -ADP. Incubation was at 37° in the nephelometer. After the times indicated, each incubation mixture was worked up as described in the text, and after chromatography in solvent system 1 (Table 4) the radioactivities of the separated substances were determined. ■, ATP; ●, ADP; ○, AMP; △, adenosine + hypoxanthine; ▲, inosine. —, Heparin-citrate; ----, heparin-saline. Results are not corrected for recovery of substances from charcoal.

± 1.1 min. (s.d.) and 10.95 min. ± 1.5 min. (s.d.) with heparin and heparin-citrate as anticoagulant respectively. Each incubation of the relevant heparin-treated or heparin-citrate-treated plasma was done at the same time. AMP accumulated to about the same extent in both incubation mixtures, but appeared to decay more rapidly in the presence of heparin than in the presence of heparin-citrate. Adenosine and inosine accumulated to a greater extent in the presence of heparin than of heparin-citrate. ATP accumulated sooner and to a greater extent in the presence of heparin than of heparin-citrate, although at 20–21.5 min. incubation there was probably little difference between the values obtained. Hypoxanthine in the experiment illustrated by Fig. 4 was determined together with adenosine, but visual inspection of radioautographs obtained after electrophoresis of portions of the charcoal eluates showed that hypoxanthine was only visible at 20 or 21.5 min. incubation times and was present at these times in approximately equal amount in plasmas of both types.

Incubation of ADP with human PR-plasma or PP-plasma. After adding ADP at an initial concentration of 200 μM to plasma containing 170×10^6 – 350×10^6 platelets/ml. and plasma containing 6×10^6 – 40×10^6 platelets/ml., it was found that there was no difference between the rates of breakdown of ADP or of AMP in the presence of heparin and of heparin-citrate (Ireland & Mills, 1964). These results were obtained by the method based on the specific oxidation of NADH. Chromatography and electrophoresis of charcoal eluates derived from plasmas with heparin as anticoagulant confirmed that the rates of breakdown of ADP and of AMP were the same in PR-plasma and PP-plasma. It was also shown that ATP accumulated in both PR-plasma and PP-plasma, with heparin as anticoagulant, to the extent of about 10% of the total substances recovered after 15–30 min. incubation. ATP thereafter decayed, with none remaining at 120 min. Hypoxanthine accumulated to a markedly greater extent in PR-plasma than in PP-plasma. After 4 hr. incubation, some 35% of the total recovered metabolites was present as hypoxanthine in PR-plasma, as opposed to about 6% in PP-plasma. Inosine accumulated to a greater extent in PP-plasma than in PR-plasma, but the accumulation of adenosine was the same in both plasmas up to 60 min. incubation, but after that the accumulation and then the decay of adenosine was more rapid in PR-plasma than in PP-plasma.

The results of an incubation of $[8-^{14}\text{C}]$ ADP at an initial concentration of 1.5 μM -ADP with either PR-plasma or PP-plasma in the presence of heparin are illustrated by Fig. 5. The rate of breakdown of ADP was the same in both plasmas,

with a half-life of about 6min. in the experiment illustrated by Fig. 5. The mean half-life of ADP breakdown for five incubations of ADP with PR-plasma from five men was $6.61 \text{ min.} \pm 0.75 \text{ min.}$ (s.d.) and with PP-plasma from four men was $7.69 \text{ min.} \pm 1.1 \text{ min.}$ (s.d.). Two of the above comparisons were done at the same time, with the relevant plasmas obtained on each occasion from one person. The other incubations, three with PR-plasma and two with PP-plasma, were done at different times. The amount of ATP present in PP-plasma was less than 0.5% of the total recovered substances at all incubation times, but ATP accumulated in PR-plasma from about 0.5% of total recovered substances at 2.5min. incubation to 5.3% of total recovered substances at 20min. Hypoxanthine formation was about 2.6 and 1.0% of total recovered substances at 20min. incubation in PR-plasma and PP-plasma respectively. The accumulation of the total of both AMP and inosine was the same in PR-plasma as in PP-plasma, but between 10min. and 20min. incubation adenosine accumulated to a greater extent in PP-plasma than in PR-plasma.

The results of longer times of incubation of $[8\text{-}^{14}\text{C}]\text{ADP}$ with PR-plasma are illustrated in Fig. 6. The ADP content fell to about 6% of the total recovered substances at 80min. incubation, but then remained constant or increased slightly. Adenosine, on the other hand, had practically disappeared by 160min. ATP accumulated rapidly between 20min. and 40min. incubation, but thereafter accumulated more slowly. The total of AMP and inosine accumulated to a maximum at about 40min. incubation and then decayed, and hypoxanthine accumulated throughout the incubation period.

Incubation of adenosine and inosine with human PR-plasma. To 2ml. of PR-plasma containing heparin and 248×10^6 platelets/ml. was added $10 \mu\text{l.}$ of an aqueous solution of $[8\text{-}^{14}\text{C}]\text{adenosine}$ of specific activity 14.7 mc/m-mole to give an initial concentration of $3.3 \mu\text{M}$ -adenosine. The mixture was incubated for 20min. at 37° and then separated by centrifugation ($1200g$ for 10min.) into two fractions. One fraction consisted of plasma containing 3.5×10^6 platelets/ml. and the other of cells, which were washed once with 0.5 ml. of buffered 0.9% sodium chloride, pH 7.4, containing EDTA. To a 1ml. portion of the PP-plasma obtained and to the washed cells after removal of the buffered saline were added 4.5ml. and 5ml. respectively, of 10% trichloroacetic acid containing the carrier compounds. The centrifuged extracts were put through charcoal, and the radioactive products of incubation were separated by both chromatography and electrophoresis. ATP, ADP, AMP and hypoxanthine were present in the fraction

Fig. 5. Incubation of $[8\text{-}^{14}\text{C}]\text{ADP}$ added either to plasma containing 175×10^6 platelets/ml. or to plasma containing 8×10^6 platelets/ml. Heparin was present in both plasmas. The plasmas were obtained from the same person on the same day. To 5ml. of each of the plasmas was added $50 \mu\text{l.}$ of aqueous $[8\text{-}^{14}\text{C}]\text{ADP}$ solution to give an initial concentration of $1.5 \mu\text{M}$ -ADP. Incubation was at 37° with mechanical shaking. At the times indicated, 1ml. samples were removed from the incubation mixtures and worked up as described in the text. After electrophoresis (Table 4) the radioactivities of the separated substances were determined. ■, ATP; ●, ADP; ○, AMP + inosine; △, adenosine; □, hypoxanthine. —, PP-plasma; ----, PR-plasma. Results are not corrected for recovery of substances from charcoal.

Fig. 6. Incubation of $[8\text{-}^{14}\text{C}]\text{ADP}$ with PR-plasma containing heparin. The initial concentration of $[8\text{-}^{14}\text{C}]\text{ADP}$ in plasma was $1.4 \mu\text{M}$. Otherwise conditions were as given in Fig. 5. ■, ATP; ●, ADP; ○, AMP + inosine; △, adenosine; □, hypoxanthine.

derived from the platelets, whereas that derived from the PP-plasma contained only adenosine, inosine and hypoxanthine. Calculation from the radioactivities obtained indicated that about 9–12, 4–7 and 0.2–0.9% of the recovered radioactivity was present in the platelets as ATP, ADP and AMP respectively after the 20min. incubation period. The corresponding value for hypoxanthine was about 0.7%. The PP-plasma contained about 54, 18 and 7% of the recovered radioactivity as inosine, adenosine and hypoxanthine respectively. Little or no ATP, ADP or AMP was detected when [$8\text{-}^{14}\text{C}$]adenosine was incubated for 20min. with PP-plasma.

PR-plasma containing 242×10^6 platelets/ml., to which [$8\text{-}^{14}\text{C}$]inosine at an initial concentration of $0.6 \mu\text{M}$ was added, was incubated for 5, 10, 20 and 40min. At these times samples were removed and analysed by the charcoal-electrophoresis method. No ATP or ADP was detected.

Incubation of adenosine and inosine with human washed platelets. Platelets washed once and resuspended in buffered 0.9% sodium chloride, pH 7.4, to which K^+ , Ca^{2+} , Mg^{2+} and glucose were added, were incubated with [$8\text{-}^{14}\text{C}$]adenosine and with [$8\text{-}^{14}\text{C}$]inosine. Portions of the incubation mixtures were removed at 15, 60 and 120min. after adding the substrates, and after charcoal treatment the radioactive products of incubation were separated by chromatography. The results are given in Table 6.

Small amounts of substances with the R_f values of ATP and of ADP were detected when inosine was incubated with the washed cells for 60 and 120min. When adenosine was incubated with the washed cells, some 60% of the radioactivity was found as ATP after 60min. and 120min. incubation and about 14 and 1.5% of the radioactivity was found as ADP and AMP respectively after 120min. incubation. In experiments similar to that described for PR-plasma, in which platelets were separated after incubation with [$8\text{-}^{14}\text{C}$]adenosine from the supernatant and washed once before analysis, it was confirmed that the radioactivity found in the cell fraction was present as ATP, ADP and AMP, whereas the radioactivity in the supernatant was present as adenosine, inosine and hypoxanthine. The rate of uptake of [$8\text{-}^{14}\text{C}$]adenosine by the platelets, calculated over the first 15min. of incubation as nucleotide production, was about 12×10^{-12} moles/min./ 10^8 platelets in the experiment the results of which are given in Table 6.

Inosine and hypoxanthine, the latter detected in other experiments by means of electrophoresis, were also formed when [$8\text{-}^{14}\text{C}$]adenosine was incubated with washed cells. Hypoxanthine, but not adenosine, was detected when [$8\text{-}^{14}\text{C}$]inosine was incubated with washed cells and the products of incubation were separated by electrophoresis, so that the values in Table 6 giving the content of adenosine plus hypoxanthine for incubations with inosine probably refer to hypoxanthine only.

Table 6. *Incubation of washed platelets with [$8\text{-}^{14}\text{C}$]adenosine and [$8\text{-}^{14}\text{C}$]inosine*

To 2ml. amounts of washed-platelet suspension were added either 20 μl . of aqueous solution containing 3.9 μmoles of [$8\text{-}^{14}\text{C}$]adenosine of specific activity 28.4 mc/m-mole or 20 μl . of aqueous solution containing 5.7 μmoles of [$8\text{-}^{14}\text{C}$]inosine of specific activity 21.8 mc/m-mole. After incubation at 37° for the times indicated 0.5ml. samples were removed and analysed by the charcoal-chromatography method (solvent system 1, Table 4). The composition of the washed-platelet suspension was: 1.73ml. of platelet suspension in buffered 0.9% NaCl, pH 7.4; 0.2ml. of 0.154 M-KCl; 0.01ml. of 0.11 M- CaCl_2 ; 0.02ml. of 0.154 M- MgSO_4 ; 0.04ml. of 5.4% (w/v) glucose. The incubation mixtures contained 366×10^6 and 391×10^6 platelets/ml. for the adenosine and inosine incubations respectively. The platelets were obtained from one person and the experiment was carried out immediately after collection of the blood. Corrections were made for loss on charcoal as follows: ATP, $\times 1.3$; ADP, AMP and inosine, $\times 1.2$; adenosine plus hypoxanthine, $\times 1.4$. The ATP/ADP ratio was calculated from corrected counts/min. found for each substance at the times indicated.

Substance	Radioactivity of each substance (% of total initial radioactivity after correction)					
	Adenosine			Inosine		
	15 min.	60 min.	120 min.	15 min.	60 min.	120 min.
ATP	27.3	63.8	62.4	0.6	5.7	10.3
ADP	5.6	9.7	15.9	0.6	1.6	1.8
AMP	0.5	0.9	1.6	0.4	0.5	0.7
Inosine	3.4	5.6	4.0	77.5	42.3	19.1
Adenosine + hypoxanthine	63.3	20.0	16.1	20.8	50.0	68.1
ATP/ADP ratio	4.9	6.6	3.9	1.0	3.6	5.5

Since carrier IMP, IDP and ITP were not included in the solutions added to charcoal it is not known whether these substances were formed during the incubation of washed cells with radioactive adenosine and inosine. However, the total radioactivity recovered and corrected for the yield of the relevant compounds on charcoal (Table 6) was about 92% of the starting radioactivity, so that if inosine nucleotide was formed the amount was probably small.

Incubation of ADP with human PR-plasma in the presence of adenosine deaminase. Fig. 7 illustrates the effect of adenosine deaminase on the incubation of $[8-^{14}\text{C}]\text{ADP}$ with PR-plasma. The rates of decay of ADP were similar in the presence and absence of adenosine deaminase. Half of the ADP was eliminated by about 8.25 min. In the presence of the deaminase, less than 0.1% of the total recovered radioactivity was present as adenosine at 2, 6 and 18 min. after adding ADP, whereas without the deaminase adenosine accumulated to 31% of the total recovered radioactivity by 18 min. incubation. Inosine plus AMP were present at

18 min. incubation to the extent of 69 or 40% of the total recovered radioactivity in the presence or absence of the deaminase respectively. The amount of ATP increased from 0.6% at 6 min. to 3.2% of the total radioactivity at 18 min. with deaminase not present. In its presence trace amounts of ATP were detected, about 0.1% at 6 min. to 0.6% of the total recovered radioactivity at 18 min.

Metabolism of ADP and aggregation of platelets. The relation of the metabolism of $[8-^{14}\text{C}]\text{ADP}$ at an initial concentration of $2.5\mu\text{M}$, in human PR-plasma and in rat PR-plasma with heparin as anticoagulant, to the course of aggregation of platelets is shown in Fig. 8. The decay of ADP in rat plasma was rapid, with elimination of one-half of the ADP in about 1.3 min., as opposed to about 6 min. in human PR-plasma. In rat PR-plasma, AMP accumulated to a maximum of about 29% of the total recovered substances by about 30 sec. and then decayed to 8% of the total recovered substances by 5 min. Adenosine accumulated to 78% of the total recovered substances by 5 min. Inosine and ATP accumulated to about 3 and 5.5% respectively of the total recovered substances during the 5 min. incubation. The results of the experiment on the metabolism of ADP in human PR-plasma in the presence of heparin (Fig. 8) were similar to those already described (Fig. 4).

The aggregation and disaggregation of platelets in human and rat plasma have been described (Cuthbertson & Mills, 1963b). In the presence of $2.5\mu\text{M}$ -ADP, the rate of aggregation and the time of maximum aggregation of both human and rat platelets appeared to be similar, but with human platelets about 15.5 min. was necessary for disaggregation, whereas with rat platelets disaggregation was practically complete 2.5 min. after maximum aggregation (Fig. 8).

DISCUSSION

The quantitative results obtained (Figs. 2 and 3) when ADP at initial concentrations of about $200\mu\text{M}$ was incubated with human plasma should be treated with some reservations. The amount of each substance present varied with the time of incubation and as shown in Table 1 the recovery of these substances from charcoal varied with the amount applied. Moreover, the recovery of each substance may be influenced by the presence of the other substances, and in a complicated mixture that is not constant in composition at all incubation times the recovery of each substance is difficult to determine. With the use of radioactive ADP, at an initial concentration of 0.5 – $2.5\mu\text{M}$ -substrate, these difficulties could be overcome, since constant amounts of the same carrier compounds were added to extracts of the incubation mixtures and

Fig. 7. Effect of adenosine deaminase on products of incubation of $[8-^{14}\text{C}]\text{ADP}$ with plasma containing 256×10^6 platelets/ml. and heparin. To one 4 ml. portion of plasma $40\mu\text{l.}$ of deaminase preparation (see the text) was added and $40\mu\text{l.}$ of $2.8\text{M}-(\text{NH}_4)_2\text{SO}_4$ solution to another 4 ml. of plasma. After 5 min. incubation at 37° , $40\mu\text{l.}$ of aqueous $[8-^{14}\text{C}]\text{ADP}$ solution was added to both incubation mixtures, to give an initial concentration of $1.35\mu\text{M}$ -ADP. Otherwise conditions were as given in Fig. 5. ■, ATP; ●, ADP; ○, AMP+inosine; △, adenosine; □, hypoxanthine. —, With adenosine deaminase; ----, without adenosine deaminase. The amounts of adenosine and ATP in the presence of adenosine deaminase were less than 0.09 and 0.6% respectively of the total recovered radioactivity and are not illustrated.

Fig. 8. Comparison between incubation of $[8-^{14}\text{C}]\text{ADP}$ at an initial concentration of $2.5\mu\text{M}$ in (a) rat PR-plasma containing heparin and (b) human PR-plasma containing heparin. Aqueous $[8-^{14}\text{C}]\text{ADP}$ solution in $2.5\mu\text{l}$. amounts was added to 0.5ml . of plasma in the nephelometer. Incubation was at 37° . The reactions were stopped with trichloroacetic acid at the time indicated, and each incubation mixture was worked up as described in the text. After chromatography with solvent system 1 and electrophoresis (Table 4) the radioactivities of the separated substances were determined. ■, ATP; ●, ADP; ○, AMP; △, adenosine; ▲, inosine. Hypoxanthine was not detected by radioautography and was not determined. Results are not corrected for recovery of the substances on charcoal. The lower curves represent the light-transmittance of the PR-plasma during the incubations and results are expressed as percentages of the maximum value obtained after the addition of ADP. The two curves represent incubations that were sampled at the times indicated.

the recovery of each compound from charcoal under these conditions was empirically determined (Table 3). It was, however, necessary to know the identity of the substances formed during the incubation of ADP with human plasma so that the relevant carrier compounds could be included.

ATP, ADP, AMP, adenosine, inosine and hypoxanthine were detected during incubation of human plasma with ADP at an initial concentration of about $200\mu\text{M}$ (Figs. 2 and 3), and the results obtained for ADP and AMP were in reasonable agreement with the results of determinations of these substances made by enzyme methods (Fig. 1). The formation of adenine cannot be precluded since the recovery from charcoal of this compound, particularly at low levels of application, was very poor (Tables 1 and 3), but the total amount of all substances recovered was about 80–90% of the initial amount of ADP added (Figs. 2 and 3), a value that might be expected from the results given in Table 1, so that it is unlikely that much adenine was formed.

Jørgensen (1956) found that adenosine, inosine, AMP and IMP, each separately added to human plasma to give an initial concentration of each compound of $200\mu\text{M}$, were converted into hypoxanthine at about the same rate. Adenine was not converted into hypoxanthine. He concluded that the formation of hypoxanthine from adenosine occurred by way of inosine. The results illustrated in Figs. 2 and 3 support this conclusion. It is also likely, since IMP was not detected, that the route of breakdown of AMP in human plasma is mainly by way of adenosine and inosine to hypoxanthine.

Since ATP accumulated as well as AMP during the first 15–30 min. of incubation, after ADP at an initial concentration of $200\mu\text{M}$ had been added to PP-plasma, the initial breakdown of ADP may be due to myokinase ($2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$). However, when small concentrations of ADP were incubated with PP-plasma, ATP formation was less than 0.5% of the total recovered substances (Fig. 5), and this ATP may have been formed from adenosine in the few platelets present in PP-plasma.

(see below). The failure to detect ATP in PP-plasma after adding small amounts of ADP might be due to the breakdown of any ATP formed, or it might be due to the presence of another enzyme, with a lower Michaelis constant for ADP, that dephosphorylates ADP without formation of ATP. The time for eliminating half the added ADP changed from about 20 min. to about 7.7 min. when the initial concentration of ADP was decreased from 200 to $1.5 \mu\text{M}$ in PP-plasma.

Although ATP did not accumulate in PP-plasma after the addition of small amounts of ADP, it did accumulate in PR-plasma (Figs. 5 and 6). Also ADP did not, in the presence of platelets, completely disappear on prolonged incubation, whereas adenosine did (Fig. 6). It therefore seemed possible that adenosine was being phosphorylated to ADP and ATP in the platelet. Incubation of adenosine with both PR-plasma and washed-platelet suspensions for 15–120 min. showed that AMP, ADP and ATP were formed by the platelets within this time (Table 6). These substances were only found in the plasma supernatant in trace amounts after removal of the cells. Similar incubations with inosine added to washed-cell suspensions caused the formation of small amounts of ATP and ADP after 1 and 2 hr. incubation (Table 6). The results given in Fig. 7 show that the accumulation of ATP is decreased to a very small amount if, by the action of added adenosine deaminase, adenosine is prevented from accumulating during the incubation of ADP with PR-plasma, thus indicating that ADP and AMP do not penetrate the platelet membrane. However, traces of ATP appeared to be formed and the origin of this material is not known. The platelet membrane thus appears to be permeable to adenosine, which is in part converted by cellular mechanisms into AMP, ADP and ATP, and these substances are retained within the platelet.

The platelets, as well as accumulating phosphorylated derivatives of adenosine, convert adenosine into inosine and convert inosine into hypoxanthine (Table 6 and the text).

The deamination of adenosine to inosine occurs in PP-plasma, and the presence of platelets does not appear to enhance the formation of inosine (Fig. 5). The formation of hypoxanthine, however, was greater in PR-plasma than in PP-plasma (see the text). The formation of hypoxanthine from inosine in plasma has been discussed by Jørgensen (1956) and has been ascribed to the action of nucleoside phosphorylase (Kalckar, 1945) and also to the presence in plasma of an enzyme capable of splitting ribose 1-phosphate. Presumably the platelets also contain these enzymes.

There was little difference between the rates of decay of ADP and AMP in the presence or absence of platelets (Ireland & Mills, 1964; see also the text and Fig. 5), indicating either that platelets do not contribute to the breakdown of these substances, or, if they do, that their activity is small compared with that of plasma. In this connexion, Jørgensen (1956) found that the breakdown of ATP was much more rapid in whole blood than in plasma. It is not yet known whether platelets contribute to the breakdown of ATP or whether erythrocytes contribute to the breakdown of ADP and AMP.

The rate of decay of ADP in either PR-plasma or PP-plasma was about 2.2 times as fast in the presence of heparin as in the presence of heparin-citrate when the initial concentration of ADP incubated was about $200 \mu\text{M}$ (Ireland & Mills, 1964; and the text). At an initial concentration of $1.2 \mu\text{M}$ -ADP, however, the rate of decay was only about 1.3 times as fast in the presence of heparin as in the presence of heparin-citrate (see the text). The difference between these ratios may also indicate that at low initial ADP concentration the enzyme(s) degrading ADP is not the same as at the higher substrate concentration.

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